



## VERIFICATION OF TRANSLATION

I, Mikiko Oyanagi

of 6th Fl., Kantetsu Tsukuba-Science-City Bldg. 1-1-1, Oroshi-machi, Tsuchiura, Ibaraki,  
JAPAN

declare as follows:

1. That I am well acquainted with both the English and Japanese languages, and
2. That the attached document is a true and correct translation made by me to the best of my knowledge and belief of:

- (a) Japanese Patent Application No. Hei 11-131978  
Entitled: "BLOOD CARBONYL COMPOUND-TRAPPING AGENT"  
Filed on May 12, 1999

October 28, 2004  
(Date)

M Oyanagi  
(Signature of Translator)  
Mikiko Oyanagi

**【Name of Document】 APPLICATION FOR PATENT**

**【Identification Number】 KRK-105**

**【Filing Date】 May 12, 1999**

**【Addressee】 Esq. Commissioner of the Patent Office**

**【International Patent Classification】 A61M 1/14**

**【Inventor】**

**【Address】 4-2-3-101 Higashinaruse, Isehara-shi, KANAGAWA**

**【Name】 Toshio Miyata**

**【Applicant】**

**【Identification Number】 597142376**

**【Name or Appellation】 Toshio Miyata**

**【Applicant】**

**【Identification Number】 597142387**

**【Name or Appellation】 Kiyoshi Kurokawa**

**【Attorney】**

**【Identification Number】 100102978**

**【Patent Attorney】**

**【Name or Appellation】 Hatsushi Shimizu**

**【Nominated Attorney】**

**【Identification Number】 100108774**

**【Patent Attorney】**

**【Name or Appellation】 Kazunori Hashimoto**

**【Payment】**

**【Registration Number】 041092**

**【Amount】 21000**

**【List of Attached Documents】**

**【Name of Document】 Description 1**

**【Name of Document】 Drawings 1**

**【Name of Document】 Abstract 1**

[Document Name] Description

[Title of the Invention] BLOOD CARBONYL COMPOUND-TRAPPING AGENT

[Claims]

5 [Claim 1] A carrier to which a carbonyl compound-trapping agent is immobilized, wherein said carrier removes a blood carbonyl compound.

[Claim 2] The carrier according to claim 1, which is used in hemodialysis.

10 [Claim 3] The carrier according to claim 2, which is a dialysis membrane.

[Claim 4] The carrier according to claim 3, wherein said dialysis membrane is a polysulfone membrane.

15 [Claim 5] The carrier according to any one of claims 1 to 4, wherein said carbonyl compound-trapping agent is aminoguanidine, pyridoxamine, hydrazine, or SH group-containing compound, or derivatives thereof.

[Claim 6] The carrier according to any one of claims 1 to 4, wherein said carbonyl compound-trapping agent is a Maillard reaction inhibitor.

20 [Claim 7] An agent for improving the carbonyl stress state in blood, wherein said agent comprises a carbonyl compound-trapping agent as an active ingredient.

25 [Claim 8] The agent for improving the carbonyl stress state according to claim 7, wherein said agent is immobilized within the blood circuit.

[Claim 9] The agent for improving the carbonyl stress state according to claim 7 or 8, wherein said carbonyl compound-trapping agent is aminoguanidine, pyridoxamine, hydrazine, or SH group-containing compound, or derivatives thereof.

30 [Claim 10] The agent for improving the carbonyl stress state according to claim 7 or 8, wherein the carbonyl compound-trapping agent is a Maillard reaction inhibitor.

35 [Claim 11] A method for improving carbonyl stress state, wherein said method comprises the step of contacting, within the blood circuit, a patient's blood with a carbonyl compound-trapping agent.

[Claim 12] The method according to claim 11, wherein said method

comprises the step of immobilizing said carbonyl compound-trapping agent on a carrier.

[Detailed Description of the Invention]

[0001]

5 [Technical Field of Industrial Application]

The present invention relates to the removal of blood carbonyl compounds, specifically, to the removal of blood carbonyl compounds using a carrier to which a carbonyl compound-trapping agent is immobilized.

10 [0002]

[Prior Art]

Hemodialysis is a typical therapy used for treating patients with chronic renal failure, wherein blood waste products and toxic substances are removed by contacting blood with a dialysate via a semipermeable membrane. However, the disease state of renal failure cannot completely be inhibited by dialysis. Such a disease state includes increase of advanced glycation end product (AGE) levels in renal failure patients. AGEs modify protein structure and function and contribute the onset of complications of dialysis, such as dialysis amyloidosis and arteriosclerosis (Makita, Z. et al., N. Engl. J. Med., 325: 836-842, 1991; Miyata, T. et al., J. Clin. Invest., 93: 521-528, 1994). Recently, it was revealed that the accumulation of carbonyl AGE precursors in blood plasma results in an increased AGE level in renal failure (Miyata, T. et al., J. Am. Soc. Nephrol., 9: 2349-2356, 1998; Miyata, T. et al., Kidney Int., 55: 389-399, 1999). A variety of these carbonyl intermediates derive from carbohydrates and lipids (Miyata, T. et al., Kidney Int., 55: 389-399, 1999; Miyata, T. et al., Kidney Int., 54: 1290-1295, 1998; Miyata, T. et al., Kidney Int., 51: 1170-1181, 1997). Conventional hemodialysis cannot effectively remove these increased levels of AGE and carbonyl intermediates, namely "carbonyl stress", in renal failure patients.

[0003]

[Problems to Be Solved by the Invention]

35 An objective of the present invention is to provide a carrier to which a carbonyl compound-trapping agent is immobilized for

removing blood carbonyl compounds. Another objective of the present invention is to provide a method and agent for improving carbonyl stress state in blood. The present invention enables to prevent the damage caused by carbonyl compounds in hemodialysis patients. The objective of the present invention is to reduce the damage by carbonyl compounds as much as possible in hemodialysis patients.

[0004]

[Means to Solve the Problem]

First, the present inventors studied how the hemodialysis membrane used for hemodialysis influenced the quantity of carbonyl compounds in patients' blood. The content of blood pentosidine, a marker for the carbonyl precursors (i.e. carbonyl stress), was compared by quantification with high-performance liquid chromatography (HPLC) for each type of dialysis membrane used by dialysis patients. The result showed that free pentosidine was markedly removed by dialysis through any dialysis membrane, while protein-bound pentosidine, which occupies the major portion of pentosidine in the body could not be effectively removed by dialysis.

[0005]

Comparing the type of dialysis membrane indicated no difference in the value of protein-bound and free pentosidine with low-flux cellulosic, high-flux polymethyl methacrylate (PMMA) and AN69, but the value was lower with high-flux polysulfone (PS) ( $p < 0.01$ ). There were no differences of the value depending on whether the patient was Japanese or Belgian, or on the manufacturer of the PS membrane. Switching the dialysis membrane used by three patients from AN69 to PS decreased the protein-bound pentosidine level, and then, return to AN69 increased the level back to the original. These results revealed that polysulfone membrane is effective as a dialysis membrane for suppressing the generation of carbonyl compounds.

[0006]

Then, the present inventors conceived the utilization of a carbonyl compound-trapping agent for the effective removal of blood carbonyl compounds. Blood plasma prepared from the blood of

dialysis patients was incubated with carriers on which a carbonyl compound-trapping agent had been immobilized, and blood carbonyl compounds were quantified. The result showed that the incubation with carriers on which a carbonyl compound-trapping agent had been  
5 immobilized significantly reduced the quantities of blood carbonyl compounds.

[0007]

Based on these findings, the present inventors focused on carbonyl compounds accumulated in blood and found that the  
10 improvement of carbonyl stress, mainly protein modification, in dialysis patients is useful. Then, the inventors found that the use of a carrier to which a compound eliminating or reducing the protein modification activity of carbonyl compounds by chemically reacting with or adsorbing carbonyl compounds is immobilized was  
15 effective to achieve the objective, and thus completed the present invention. In the present invention, such compound is called a "carbonyl compound-trapping agent".

[0008]

Namely, the present invention relates to a carrier to which  
20 a carbonyl compound-trapping agent is immobilized for removing blood carbonyl compounds, as well as a method and agent for improving carbonyl stress state in blood. Specifically, the present invention relates:

[1] a carrier to which a carbonyl compound-trapping agent is  
25 immobilized, wherein said carrier removes a blood carbonyl compound;

[2] the carrier according to [1], which is used in hemodialysis;

[3] the carrier according to [2], which is a dialysis membrane;

[4] the carrier according to [3], wherein said dialysis membrane  
30 is a polysulfone membrane;

[5] the carrier according to any one of [1] to [4], wherein said carbonyl compound-trapping agent is aminoguanidine, pyridoxamine, hydrazine, or SH group-containing compound, or derivatives thereof;

[6] the carrier according to any one of [1] to [4], wherein said  
35 carbonyl compound-trapping agent is a Maillard reaction inhibitor;

[7] an agent for improving the carbonyl stress state in blood,

wherein said agent comprises a carbonyl compound-trapping agent as an active ingredient;

[8] the agent for improving the carbonyl stress state according to [7], wherein said agent is immobilized within the blood circuit;

5 [9] the agent for improving the carbonyl stress state according to [7] or [8], wherein said carbonyl compound-trapping agent is aminoguanidine, pyridoxamine, hydrazine, or SH group-containing compound, or derivatives thereof;

10 [10] the agent for improving the carbonyl stress state according to [7] or [8], wherein the carbonyl compound-trapping agent is a Maillard reaction inhibitor;

[11] a method for improving carbonyl stress state, wherein said method comprises the step of contacting, within the blood circuit, a patient's blood with a carbonyl compound-trapping agent; and

15 [12] the method according to [11], wherein said method comprises the step of immobilizing said carbonyl compound-trapping agent on a carrier.

[0009]

[Mode for Carrying Out the Invention]

20 In the present invention, carbonyl compounds to be trapped include, for example, the following compounds that accumulate in the blood of renal failure patients together with oxidative stress.

[0010]

Carbonyl compounds derived from carbohydrates:

- 25
- arabinose
  - glyoxal
  - methylglyoxal
  - 3-deoxyglucosone

Carbonyl compound derived from ascorbic acid:

- 30
- dehydroascorbic acid

Carbonyl compound derived from lipid:

- hydroxynonenal
- malondialdehyde
- acrolein

35 [0011]

A preferred carbonyl compound-trapping agent in the present

invention is one capable of completely inhibiting or reducing the protein-modification activity of all these carbonyl compounds through a chemical reaction or adsorption. However, the carbonyl compound-trapping agent of the present invention also includes an agent effective for the major carbonyl compounds among these. Carbonyl compound-trapping agents that can be used in the present invention include, for example, the following compounds:

[0012]

• aminoguanidine (Foote, E. F. et al., Am. J. Kidney Dis., 25: 420-425 (1995))

•  $\pm$  2-isopropylidenehydrazono-4-oxo-thiazolidin-5-yl-acetanilide (OPB-9195; S. Nakamura, 1997, Diabetes 46:895-899)

[0013]

Further, the carbonyl compound-trapping agent includes, for example, the following compounds or derivatives thereof that are capable of functioning as carbonyl compound-trapping agents. "Derivatives" indicate compounds having an atomic or molecular substitution(s) at any position as compared with the parent compound.

[0014]

(1) guanidine derivatives such as methylguanidine (JP-A Sho 62-142114; JP-A Sho 62-249908; JP-A Hei 1-56614; JP-A Hei 1-83059; JP-A Hei 2-156; JP-A Hei 2-765; JP-A Hei 2-42053; JP-A Hei 6-9380; Published Japanese Translation of International Publication 5-505189), etc.

(2) hydrazine derivatives such as sulfonylhydrazine, etc.

(3) five-membered heterocyclic compounds having two nitrogen atoms, such as pyrazolone (JP-A Hei 6-287179), pyrazoline (JP-A Hei 10-167965), pyrazole (JP-A Hei 6-192089; JP-A Hei 6-298737; JP-A Hei 6-298738), imidazolidine (JP-A Hei 5-201993; JP-A Hei 6-135968; JP-A Hei 7-133264; JP-A Hei 10-182460), hydantoin (JP-A Hei 6-135968), etc.

(4) five-membered heterocyclic compounds having three nitrogen atoms, such as triazole (JP-A Hei 6-192089), etc.

(5) five-membered heterocyclic compounds having a nitrogen atom and a sulfur atom, such as thiazoline (JP-A Hei 10-167965),



thiazole (JP-A Hei 4-9375; JP-A Hei 9-59258), thiazolidine (JP-A Hei 5-201993; JP-A Hei 3-261772; JP-A Hei 7-133264; JP-A Hei 8-157473), etc.

5 (6) five-membered heterocyclic compounds having a nitrogen atom and an oxygen atom, such as oxazole (JP-A Hei 9-59258), etc.

(7) nitrogen-containing six-membered heterocyclic compounds such as pyridine (JP-A Hei 10-158244; JP-A Hei 10-175954), and pyrimidine (Published Japanese Translation of International Publication 7-500811), etc.

10 (8) nitrogen-containing condensed heterocyclic compounds such as indazole (JP-A Hei 6-287180), benzimidazole (JP-A Hei 6-305964), quinoline (JP-A Hei 3-161441), etc.

(9) sulfur- and nitrogen-containing condensed heterocyclic compounds such as benzothiazole (JP-A Hei 6-305964), etc.

15 (10) sulfur-containing condensed heterocyclic compound such as benzothiophene (JP-A Hei 7-196498), etc.

(11) oxygen-containing condensed heterocyclic compounds such as benzopyran (JP-A Hei 3-204874; JP-A Hei 4-308586), etc.

20 (12) nitrogenous compounds such as carbazoyl (JP-A Hei 2-156; JP-A Hei 2-753), carbazic acid (JP-A Hei 2-167264), hydrazine (JP-A Hei 3-148220), etc.

(13) quinones such as benzoquinone (JP-A Hei 9-315960), and hydroquinone (JP-A Hei 5-9114), etc.

25 (14) aliphatic dicarboxylic acids (JP-A Hei 1-56614; JP-A Hei 5-310565)

(15) silicone containing compounds (JP-A Sho 62-249709)

(16) organic germanium compounds (JP-A Hei 2-62885; JP-A Hei 5-255130; JP-A Hei 7-247296; JP-A Hei 8-59485)

30 (17) flavonoids (JP-A Hei 3-240725; JP-A Hei 7-206838; JP-A Hei 9-241165; WO 94/04520)

(18) alkylamines (JP-A Hei 6-206818; JP-A Hei 9-59233; JP-A Hei 9-40626; JP-A Hei 9-124471)

35 (19) amino acids (Published Japanese Translation of International Publication 4-502611; Published Japanese Translation of International Publication 7-503713)

(20) aromatic compounds such as ascochlorin (JP-A Hei

6-305959), benzoic acid (WO 91/11997), pyrrolo-naphthyridinium (JP-A Hei 10-158265), etc.

(21) polypeptides (Published Japanese Translation of International Publication 7-500580)

5 (22) vitamins such as pyridoxamine (WO 97/09981), etc.

(23) SH group-containing compounds such as glutathione.

(24) SH group-containing proteins such as reduced albumin, etc.

(25) tetracyclines (JP-A Hei 6-256280)

10 (26) chitosans (JP-A Hei 9-221427)

(27) tannins (JP-A Hei 9-40519)

(28) quaternary ammonium ion-containing compounds

[0015]

The above-described compounds are generally known as Maillard  
15 reaction inhibitors. Maillard reaction means a non-enzymatic glycation reaction between a reducing sugar such as glucose, and an amino acid or protein. Focusing on a phenomenon of brown coloration in a mixture consisting of amino acid and reducing sugar upon heating, Maillard reported this reaction in 1912 (Maillard,  
20 L. C., Compt. Rend. Soc. Biol., 72: 599 (1912)). Maillard reaction is involved in brown coloration of food, generation of aromatic components and taste, and protein denaturation during heating or storage. Therefore, this reaction has been mainly studied in the field of food chemistry.

25 [0016]

In 1968, glycated hemoglobin (HbA<sub>1c</sub>), a micro fraction of hemoglobin, was identified *in vivo*, which was found to increase in patients with diabetes (Rahbar. S., Clin. Chim. Acta, 22: 296 (1968)). These findings helped launch a wave of interest in the significance  
30 of the *in vivo* Maillard reaction and the participation of the reaction in the onset of adult diseases, such as diabetic complications and arteriosclerosis as well as the progress of aging. Agents inhibiting the *in vivo* Maillard reaction were explored intensively, resulting in the discovery of the above-mentioned compounds as agents inhibiting  
35 the Maillard reaction.

[0017]

However, it was not known that such Maillard reaction inhibitors are capable of improving the carbonyl-stress state in peritoneal-dialysis patients by eliminating carbonyl compounds from the blood.

5 [0018]

There is no particular limitation on the type of carrier to be used for the immobilization of the carbonyl compound-trapping agent of the present invention, as long as it is harmless to the human body, and is also safe and stable as a material directly  
10 contacting blood. Such carriers include, for example, synthetic or naturally occurring organic polymer compounds, inorganic materials such as glass beads, silica gel, alumina, and activated carbon, and those of which surfaces are coated with polysaccharide or synthetic polymer.

15 [0019]

A carrier comprising a polymer compounds is exemplified by a polymethyl methacrylate, polyacrylonitrile, polysulfone, vinyl, polyolefin, fluorine, polyester, polyamide, polyimide, polyurethane, polyacryl, polystyrene, polyketone, silicon,  
20 cellulose, chitosan; specifically, polysaccharides such as agarose, cellulose, chitin, chitosan, sepharose, dextran, etc. and derivatives thereof, and polyester, polyvinyl chloride, polystyrene, polysulfone, polyethersulfone, polypropylene, polyvinyl alcohol, polyarylether sulfone, polyacrylic ester, polymethacrylic ester, polycarbonate, acetylated cellulose,  
25 polyacrylonitrile, polyethylene terephthalate, polyamide, silicone resin, fluororesin, polyurethane, polyetherurethane, and polyacrylamide and derivatives thereof. These polymer materials can be used alone or in a combination of two or more types of polymers.  
30 In the latter case, the carbonyl compound-trapping agent is immobilized on at least one of the polymers. The immobilized carbonyl compound-trapping agent is used alone or in a combination of two or more types of compounds.

[0020]

35 There is no restriction on the shape of carrier. For example, the carrier can be membrane-like, fiber-like, granular-shaped,

hollow fiber-like, non-woven fabric-like, porous, or honeycomb-shaped. The carrier's area of contact with the peritoneal dialysate can be controlled by varying the thickness, surface area, diameter, length, shape, and/or size of the carrier.

5 [0021]

The carbonyl compound-trapping agent can be immobilized on the above-mentioned carrier by using known methods, such as physical adsorption, specific biochemical binding reaction, ion binding, covalent bonding, grafting, etc. If necessary, a spacer can be  
10 inserted between the carrier and the carbonyl compound-trapping agent. When the carbonyl compound-trapping agent is toxic, the amount released becomes a vital issue. Thus, it is preferred that the carbonyl compound-trapping agent is immobilized on the carrier by a covalent bond so as to minimize the amount released. Functional  
15 groups on the carrier are utilized for covalently bonding the carbonyl compound-trapping agent thereto. The functional group used is, for example, hydroxyl group, amino group, aldehyde group, carboxyl group, thiol group, silanol group, amide group, epoxy group, succinylimino group, etc.; however, the functional group is not  
20 limited to these groups. As examples of covalent bonds, ester bond, ether bond, amino bond, amid bond, sulfide bond, imino bond, disulfide bond, or the like can be given.

[0022]

A commercially available product, for example polystyrene  
25 carrier having sulfonylhydrazine groups (PS-TsNHNH<sub>2</sub>, ARGONAUT TECHNOLOGIES CO.), can be used as a carrier for immobilizing carbonyl compound-trapping agent.

[0023]

The carrier with the immobilized carbonyl compound-trapping  
30 agent of the present invention can be sterilized by an appropriate sterilization method selected from known sterilization methods depending upon the types of carbonyl compound-trapping agent and carrier used. The sterilization method includes, for example, autoclaving, gamma-ray irradiation, gas sterilization, etc.

35 [0024]

Carbonyl compound-trapping agent-immobilized carriers could

be contacted with blood in various ways. Examples are: the method where collected patient blood is infused into a blood bag filled with carbonyl compound-trapping agent-immobilized carriers, and trapping the carbonyl compounds in patient blood; the method where  
5 blood is circulated in a column filled with bead carriers or fiber carriers, or the like, on which a carbonyl compound-trapping agent has been immobilized, etc. Not only whole blood, but also separated blood plasma may be used for the treatment. The treated blood may be returned to the patient or, if required, may be stored in a blood  
10 bag, or the like. It is also possible to trap carbonyl compounds that generate and accumulate during storage, by including carriers on which carbonyl compound-trapping agents are immobilized within the blood bags.

[0025]

15 The contact between blood and carriers on which a carbonyl compound-trapping agent of this invention has been immobilized can be carried out during the blood purification step, including hemodialysis, blood filtration, blood filtration dialysis, blood adsorption, and blood plasma separation.

20 [0026]

For example, both hemodialysis and trapping of carbonyl compounds can be carried out simultaneously in hemodialysis patients, by placing carriers on which a carbonyl compound-trapping agent has been immobilized in the hemodialysis circuit. In this  
25 case, it is preferable to immobilize a carbonyl compound-trapping agent on the hemodialysis membrane. Known types of dialysis membranes can be used as carriers. Examples are, cellulose derivatives such as regenerated cellulose, and cellulose triacetate; and polymethyl methacrylate, polyolefin, polysulfone,  
30 polyacrylonitrile (PAN), polyamide, polyimide, polyether nylon, silicon, and polyester copolymers, but are not limited thereto. As shown in the Examples, when polysulfone is used as the dialysis membrane, there was a decrease in the carbonyl precursor (pentosidine) level. Thus, among the above-mentioned dialysis  
35 membranes, it is particularly preferable to use a polysulfone membrane as the carrier. Instead of using a dialysis membrane as

a carrier, a column filled with carriers on which a carbonyl compound-trapping agent has been immobilized may indeed be placed in the hemodialysis circuit as described above. Through contacting a patient's blood with carriers on which a carbonyl compound-trapping agent has been immobilized, carbonyl compounds are trapped from the blood, their damaging activity towards the living body is eliminated, and they become nontoxic. An anticoagulant may be used together to prevent blood-clotting in an extracorporeal circulation. Such anticoagulants include, for example, heparin, low-molecular-weight heparin, and Futhan. These may be immobilized on carriers.

[0027]

It is predicted that there maybe some cases where carbonyl compounds in patient blood are not completely treated during dialysis if the quantity of trapping agent used during the contact with blood is too small. Since pre-determination of the quantity of carbonyl compounds in the patient blood is particularly difficult, it is effective to maintain the activity of as many trapping agents as possible within a range that ensures the safety of the patient. The dose of a trapping agent can be adjusted by altering the quantity of trapping agent immobilized on the carriers, or the dose of carriers on which the trapping agent has been immobilized.

[0028]

In addition to the organic compounds represented by the above Maillard reaction inhibitors, polymer compounds such as ion exchange resin, or inorganic compounds such as activated carbon, silica gel, alumina, and calcium carbonate can also be used as carbonyl compound-trapping agents of the present invention. These compounds, which are known as filling agents used for chromatography, can trap carbonyl compounds due to their adsorption capability. Such compounds themselves can function as carriers, and therefore, for example, a filtrator installed within an extracorporeal blood circulation circuit can be filled with them for use. Such a compound can also be utilized as a "carbonyl compound-trapping agent" comprised in the an agent for improving carbonyl stress state of the present invention. In this case, such compounds themselves

function as carriers on which a carbonyl compound-trapping agent has been immobilized as described above.

[0029]

[Examples]

5           The present invention is specifically illustrated below with reference to Examples, but it is not to be construed as being limited thereto.

[0030]

10       [Example 1] The influence of the type of hemodialysis membrane on blood plasma pentosidine level

1. Patients

          Belgian patients (n= 29) and Japanese patients (n= 97), 126 patients (69 males and 57 females) in total, who had been subjected to hemodialysis 3 times a week, were tested. They were  $61.2 \pm 13$  (standard deviation) years old on average. Only two of them were affected with mild type-II diabetes mellitus. Each patient used the same type of hemodialysis membrane for at least 3 months (or, 2 or 3 patients used the same type of membrane for less than 3 months, but ever since initiating hemodialysis). In 26 of 29 Belgian patients, dialysis membranes were reused, but there was no reuse in Japanese patients. Data of residual diuresis (ml/day), surface area of dialysis membrane, and duration of hemodialysis were obtained from clinical records of each patient.

[0031]

25   2. Types of membranes

          The following hemodialysis membranes were used: high-flux (UF index>10 ml/mmHg/h) AN69 (Hospal; France) (group of AN69); high-flux polysulfone (Fresenius; Germany) (group of PS); high-flux polysulfone (Asahi Medical; Japan) (group of APS); high-flux polymethylmethacrylate (Toray; Japan) (group of PMMA); and low-flux cellulosic (Asahi Medical; Japan) (group of cellulose).

[0032]

3. Blood plasma samples

35       Blood plasma samples were collected from all the 126 patients prior to the first hemodialysis, and then from 66 patients weekly after dialysis.

All the samples were immediately subjected to centrifugal separation. The blood plasma samples frozen at  $-20^{\circ}\text{C}$  were tested as follows:

[0033]

5 4. Quantification of total pentosidine and free pentosidine

For quantification of total pentosidine, a sample (50  $\mu\text{l}$ ) was lyophilized, dissolved in 100  $\mu\text{l}$  of 6N HCl, encapsulated with nitrogen gas and then incubated at  $110^{\circ}\text{C}$  for 16 hours, neutralized with 100  $\mu\text{l}$  of 5N NaOH and 200  $\mu\text{l}$  of 0.5 M phosphate buffer (pH 7.4),  
10 subsequently filtered through a filter with a pore size of 0.5  $\mu\text{m}$ , and diluted 20 times with PBS. For quantification of the free pentosidine, the sample (50  $\mu\text{l}$ ) was mixed with an equal amount of 10% TCA, and then centrifuged at  $5000 \times g$  for 10 minutes. The supernatant was filtered through a filter with a pore size of 0.5  
15  $\mu\text{m}$  and diluted 4 times with distilled water.

Pentosidine in each of these samples was analyzed by reverse phase HPLC using a C18 reverse phase column (Waters, Tokyo, Japan) (Miyata, T. et al., J. Am. Soc. Nephrol., 7: 1198-1206, 1996). The effluent was monitored with a fluorescence detector (RF-10A;  
20 Shimadzu) at an excitation/detection wavelength =335/385 nm. A standard curve was prepared by using synthetic pentosidine.

The level of protein-bound pentosidine (pentosidine/protein) (pmol/mg protein) was calculated by [total pentosidine in blood plasma (pmol/ml) - free pentosidine (pmol/ml)]/[blood plasma  
25 protein concentration (mg/ml)].

[0034]

5. Statistical analysis between groups of patients

The respective values, including quantification of the pentosidine level, are indicated as mean  $\pm$  standard deviation or percentage (%). The data of residual diuresis was log-transformed.  
30 Through one-way analysis of variance (one-way ANOVA) (with F-test), individual data and the pentosidine level were compared between groups of hemodialysis patients using different dialysis membranes. Furthermore, the groups of dialysis membranes were compared and  
35 analyzed by using Bonferroni t-test. Through chi 2 test, the degree of residual diuresis was compared between the various groups.



[0035]

The result of cross-sectional analysis among the five groups of patients is shown in Table 1. There were no significant age-related differences among the groups. The blood plasma protein  
5 level was higher in the AN69 and cellulosic groups. In APS group, the area of the dialysis membrane was larger, and both the period of hemodialysis prior to the study and duration of a single dialysis were longer. On the other hand, residual diuresis was higher in the PS group.

[0036]

[Table 1]

The result of dialysis of five groups of patients with different dialysis membranes						
	APS n=29	PS n=28	AN69 n=15	PMMA n=25	cellulosic n=29	ANOVA P value
age(year)	58±12	61±13	64±7	61±13	64±16	NS
period of hemodialysis(year)	13.7±7.5 $\alpha$	5.4±6.8	7.8±6.7	9.2±6.5	5.5±4.4	<.001
blood plasma protein level(g/dl)	6.7±0.5**	6.3±0.7	6.8±0.6	6.2±0.3*	7.0±0.4***	<.001
blood plasma albumin level(g/dl)	3.9±0.3 $\diamond\diamond$	3.6±0.4 $\diamond$	3.9±0.4 $\Delta$	3.6±0.3	4.2±0.3***	<.001
duration of hemodialysis(hour)	4.5±0.23 $\beta$	3.98±0.40	4.13±0.23	4.04±0.48	4.00±0.00	<.001
surface area of hemodialysis membrane(m <sup>2</sup> )	1.8	1.28±0.36	1.42±0.34	1.31±0.32	1.32±0.24	ND $\neq$
residual diuresis(ml/day)						
geometric mean(SD)	47(10)	213(250)•	34(13)	47(8)	26(8)	<.001
(range)	(0-800)	(0-3050)	(0-500)	(0-400)	(0-400)	
residual diuresis(%)§	33%	50%•	20%	28%	17%	chi2 P value <.001

\*\*\* = p&lt;0.001 vs PS and PMMA group

\*\* = p&lt;0.001 vs PMMA group

\* = p&lt;0.05 vs AN69 group

 $\diamond$  = p<0.05 vs APS group $\diamond\diamond$  = p<0.01 vs PMMA and cellulose group $\Delta$  = p<0.05 vs cellulose group $\alpha$  = p<0.01 vs cellulose group $\beta$  = p<0.001 vs all other groups

● = p=0.05 vs cellulose group

 $\neq$  : The surface of the hemodialysis membrane cannot be considered as a random variable as it is fixed by the physician. It cannot therefore be compared by a statistical test.

§ : Percentage of anuria patients

[0037]

Before dialysis, the levels of protein-bound pentosidine and of free pentosidine in blood plasma were similar among the AN69, PMMA, and cellulosic groups, but were significantly lower in the  
5 PS and APS groups. There was no significant difference between the PS and APS groups (Table 2).

[0038]

[Table 2]

Pre-dialysis Pentosidine levels in the 5 membrane groups					
	APS n=29	PS n=28	AN69 n=15	PMMA n=25	cellulosic n=29
pentosidine/protein (pmol/mg protein)	16.2±4.8	15±6.1	25.4±8.4**	23.2±9.3**	21.7±6.3**
free pentosidine (pmol/ml)	32.4±11.3	41.4±22.9	76.4±28.5**	68.8±26.7**	53.7±18.2**
					ANOVA P value
					<.001
					<.001

\*\* = p&lt;0.01 vs PS and APS groups

[0039]

Univariate analysis of a variety of factors that may influence predialysis blood plasma pentosidine, indicated that residual diuresis significantly affected protein-bound and free pentosidine: the higher the residual diuresis, the lower the pentosidine level. No correlation was found between pentosidine level and blood plasma protein level, albumin level, age, or dialysis history (Table 3). Relating to polysulfone (PS and APS groups) groups, the predialysis pentosidine level in Belgian patients or Japanese patients subjected to dialysis with polysulfone membranes from Fresenius or Asahi Medical co. were similar to each other (Table 4).

[0040]

[Table 3]

Univariate analysis of the relationship between pentosidine level and potentially explanatory continuous variables: r values					
	log diuresis	Total protein	Albumin	Age	Duration
pentosidine/protein	-0.28	-0.08	0	0.14	0.03
free pentosidine	-0.36	0.01	-0.12	0.15	0.02

\*\* = p&lt;0.01

\*\*\* = p&lt;0.001

[0041]

[Table 4]

Pentosidine levels and residual diuresis in polysulfone groups, according to polysulfone brand and/or country of patients

	Fresenius Belgium	Fresenius Japan	Asahi Japan	P value
pentosidine/protein (pmol/mg protein)	14.6±6.2	15.3±6.3	16.2±4.8	NS
free pentosidine (pmol/ml)	37.3±19.6	45.4±25.9	32.4±11.3	NS
residual diuresis (ml/day)	938(23)	49(14)	47(10)	0.004

[0042]

As described above, it was revealed that pentosidine levels were lower in dialysis patients using polysulfone dialysis membranes than those in dialysis patients using other dialysis membranes. Decreases in pentosidine levels were seen in patients

dialyzed with a polysulfone membrane, independent of the patients' country or the manufacturer of the dialysis membranes. Furthermore, although patients subjected to dialysis with polysulfone dialysis membrane from Ashahi Medical were substantially anuric, pentosidine levels were similarly lower. In the group using polysulfone from Fresenius, there was no alteration in statistical significance in the difference of pentosidine level, even when patients with urinary volumes greater than 300 ml/min were excluded.

[0043]

Because AN69 is also high-flux type and pentosidine levels before dialysis in dialysis with high-flux AN69 and low-flux cellulosic showed similar results, the difference in pentosidine level is estimated to be independent of the removing capacity of dialysis membrane.

[0044]

The relationship between protein-corrected pentosidine level or free pentosidine level and residual diuresis was analyzed by linear regression analysis. The effect of each explanatory variable on dependent variable (protein-corrected pentosidine level and free pentosidine level) was tested by variable selection-multiple regression analysis (Forward stepwise multiple regression analysis). All the analyses were carried out by using BMDP statistics software (BMDP is the trade mark of New System Professional Edition: Statistical Solutions Inc., University of California Press, Berkeley, 1995). A P value less than 0.05 was considered significant.

[0045]

The analysis result showed that dialysis membrane type and residual diuresis were the two sole independent determinants of protein-bound and free pentosidine levels (Table 5). Considering the fact that none of the interactions were significant, the influence of residual diuresis on pentosidine level was not affected by dialysis membrane type.

[0046]

[Table 5]

The variable selection-multiple regression analysis of determinants of pentosidine levels  
(Forward stepwise multiple regression analysis)

	pentosidine/protein		free pentosidine	
	Increase in R	P value	Increase in R	P value
membrane type	<b>0.53</b>	<b>&lt;0.001</b>	<b>0.59</b>	<b>&lt;0.001</b>
log. diuresis	<b>-0.21</b>	<b>&lt;0.001</b>	<b>-0.36</b>	<b>&lt;0.001</b>
total protein	<b>-0.17</b>	<b>NS</b>	<b>0.05</b>	<b>NS</b>
albumin	<b>-0.05</b>	<b>NS</b>	<b>-0.1</b>	<b>NS</b>
age	<b>0.12</b>	<b>NS</b>	<b>0.16</b>	<b>NS</b>
period of hemodialysis	<b>-0.01</b>	<b>NS</b>	<b>-0.08</b>	<b>NS</b>

[0047]

- 5 6. The effect of dialysis membrane on pentosidine levels before and after hemodialysis

To further analyse the mechanism underlying dialysis membrane effect on predialysis pentosidine levels, pentosidine levels before and after dialysis were determined for four groups of patients with  
10 high-flux polysulfone (Fresenius), AN69, PMMA, or low-flux cellulosic dialysis membranes (Table 6).

[0048]

[Table 6]

Influence of a single hemodialysis session on pentosidine level

	PS n=14	AN69 n=15	PMMA n=9	cellulosic n=28
pentosidine/protein(pmol/mg protein)				
Pre	14.6±6.2	25.4±8.4	24.3±8.5	21.8±6.4
Post	13.8±6.6	23.4±5.6	22.8±8.3	22.1±5.8
free pentosidine(pmol/ml)				
Pre	37.3±19.6	76.4±28.5	70.3±26	53.5±18.5
Post	10.9±6.6	17.5±4.9	21.7±7	14.7±6.9
(reduction ratio) (%)	(71±11)	(76±7)	(67±9)	(73±6)

15 [0049]

As predicted by the above experiment, the level of protein-bound pentosidine was almost unaltered and also was independent of the dialysis membrane type. Although only the level of free pentosidine markedly decreased, the percentage, which was  
20 between 76%(AN69) and 67%(PMMA) was similar in all the groups.

There was no significant difference among the groups. Thus, it was revealed that the lower predialysis pentosidine levels observed in dialysis patients using polysulfone membranes could not be accounted for by differences in the dialysis capacity of the membranes.

[0050]

The present inventors previously showed that hemodialysis itself did not affect the total pentosidine or protein-bound pentosidine levels (Miyata, T. et al., *Kidney Int.*, 51: 880-887, 1997). This finding agrees with the fact that 95% of pentosidine bind with albumin that cannot be eliminated through dialysis (Miyata, T. et al., *J. Am. Soc. Nephrol.*, 7: 1198-1206, 1996). This finding is supported by using the above-described four different types of dialysis membranes. On the contrary, the amount of free pentosidine decreased by hemodialysis, and a similar phenomenon was observed with every dialysis membrane. This result is predictable by the molecular weight of free pentosidine (379 Da). The fact that pentosidine levels before and after hemodialysis were similar for all the dialysis membranes indicates that not only passive transport, but also absorption of pentosidine occurs during dialysis with polysulfone or other dialysis membranes. The level of absorption of radio-labeled free pentosidine with cellulosic membrane or polysulfone membrane *in vitro* is very small, and in reality, there was no difference.

[0051]

Thus, the decreased predialysis pentosidine level cannot be explained by the fact that the polysulfone membrane enhances removal of pentosidine. There may be another possibility that the dialysis with polysulfone membrane relates to the suppression of pentosidine production.

[0052]

As pointed out previously, pentosidine level reflects the concentration of carbonyl precursors derived from carbohydrates. The polysulfone membrane has the specific effect of removing these carbonyl compounds, and thus, it may be having an effect on pentosidine production. Alternatively, there may be also the



possibility that the polysulfone membrane reduces oxidative stress assumed to be associated with uraemia (Miyata, T. et al., *Kidney Int.*, 54: 1290-1295, 1998; Miyata, T. et al., *Kidney Int.*, 51: 1170-1181, 1997; Loughrey, CM. et al., *Q. J. Med.*, 87: 679-683, 1994; Ueda, Y. et al., *Biochem. Biophys. Res. Commun.*, 245: 785-790, 1998; Kumano, K. et al., *Adv. Perit. Dial.*, 1992; 8: 127-130; Witko-Sarsat, V. et al., *Kidney Int.*, 49: 1304-1313, 1996). There is also the possibility that the decrease in oxidative stress suppresses the production of carbonyl compounds, and as a result, the generation of pentosidine is decreased (Miyata, T. et al., *Kidney Int.*, 51: 1170-1181, 1997).

[0053]

7. The effect of switching the dialysis membrane on the pentosidine level

To verify the specific pentosidine-lowering effect of polysulfone, a longitudinal analysis was conducted in three anuria patients subjected to long-term (>5 years) AN69 dialysis. The patients were switched to a polysulfone (Fresenius) dialysis membrane of similar surface area for 10 weeks, and subsequently, the membrane was returned to AN69. Predialysis samples were collected every 2 weeks before the switch to PS (2 samples), during PS dialysis (5 samples), and 14-16 weeks after the return to AN69 (2 samples).

[0054]

It was found that protein-bound pentosidine level of each patient gradually decreased after switching to PS dialysis, and subsequently, due to the resumption of AN69 dialysis, the level returned to that when using AN69 prior to the switch to PS (Fig. 1).

[0055]

The decrease in pentosidine level observed in the longitudinal study in patients, which was carried out by switching AN69 to PS dialysis, is only one third of the difference (10.4 compared to 3.6 pmol/mg protein) between the PS and AN69 groups observed in the cross-sectional study. This discrepancy may be due to the fact that the observation after the transfer to PS dialysis lasted only 10

weeks. If polysulfone reduces the rate of pentosidine generation, it will be possible to explain the fact that protein-bound pentosidine decreased so slowly. Under such circumstances, the decrease in protein-bound pentosidine level may result from only protein metabolism. A similar observation was made after successful kidney transplantations. In such cases, the decrease in protein-bound pentosidine was considerably slower than the decrease in blood plasma  $\beta$ 2-microglobulin, suggesting that the decrease occurs very slowly, and the decomposition of protein-bound pentosidine is very slow (Miyata, T. et al., Kidney Int., 51: 880-887, 1997; Hricik, DE. et al., Clin. Transplantation, 10: 568-573, 1996).

[0056]

[Example 2] The removal of blood carbonyl compounds by carriers on which a carbonyl compound-trapping agent has been immobilized

A cross-linked polystyrene resin bound to a sulfonyl hydrazine group (PS-TsNHNH<sub>2</sub>, ARGONAUT TECHNOLOGIES) was used as a carbonyl compound-trapping bead to study its effect in removing blood carbonyl compounds. Blood plasma from a dialysis patient and those supplemented with carbonyl compound-trapping beads were incubated at 37°C to test the pentosidine generation-suppressing effect. 100  $\mu$ l of dimethylsulfoxide was added to the tube containing carbonyl compound-trapping beads to swell the beads, and then filter-sterilized blood plasma from a predialysis dialysis patient was added thereto. The mixture was incubated at 37°C for one week. After incubation, the beads were removed with a centrifugal filter having a pore size of 0.22  $\mu$ m (Millipore, UFC30GV00). Then, 50  $\mu$ l of 10% trichloroacetic acid was added to 50  $\mu$ l of the bead-free solution, and the mixture was centrifuged to precipitate proteins. The resulting protein pellet was washed with 300  $\mu$ l of 5% trichloroacetic acid and then dried. Subsequently, 100  $\mu$ l of 6N HCl was added to the pellet and heated at 110°C for 16 hours, and then, pentosidine was quantified by HPLC (Miyata, T. et al., 1996, J. Am. Soc. Nephrol., 7: 1198-1206, Miyata, T. et al., 1996, Proc. Natl. Acad. Sci. USA, 93: 2353-2358).

[0057]

The amount of pentosidine generated during incubation at 37°C

is shown in Fig. 2. It was found that the addition of carbonyl compound-trapping beads suppressed the generation of pentosidine. In addition, the suppression of pentosidine generation depended on the amount of carbonyl compound-trapping beads added.

5 [0058]

These results showed that blood carbonyl compounds could be removed by carriers on which the carbonyl compound-trapping agent had been immobilized. Further, it was revealed that polysulfone membrane was a particularly suitable hemodialysis membrane for  
10 improving carbonyl stress state.

[0059]

[Effects of the Invention]

The present invention enables effective removal of blood carbonyl compounds. The agent of the present invention for  
15 improving carbonyl stress state can be used readily by immobilizing it on a dialysis membrane for hemodialysis, or alternatively immobilizing it on other carriers and placing it within the blood circuit. Thus, the present invention makes it possible to ease damages caused by carbonyl compounds (i.e. carbonyl stress) from  
20 which renal failure patients have long suffered.

[Brief Description of the Drawings]

[Fig. 1]

The effect of changing the type of hemodialysis membrane on blood plasma pentosidine levels (pmol/mg protein) in 3 patients is  
25 shown. Each result is shown in a % value relative to the corresponding initial value (41.8 pmol/mg protein for patient 1 ( $\diamond$ ), 22.1 pmol/mg protein for patient 2 ( $\square$ ) or 28.5 pmol/mg protein patient 3 ( $\triangle$ )). Each value obtained is an average value of two samples collected 2 weeks after the end of each dialysis period (in  
30 the -2<sup>nd</sup> and 0<sup>th</sup> week of dialysis by AN69; in the 8<sup>th</sup> and 10<sup>th</sup> week after the switch to PS; in the 14<sup>th</sup> and 16<sup>th</sup> weeks after the returning to AN69).

[Fig. 2]

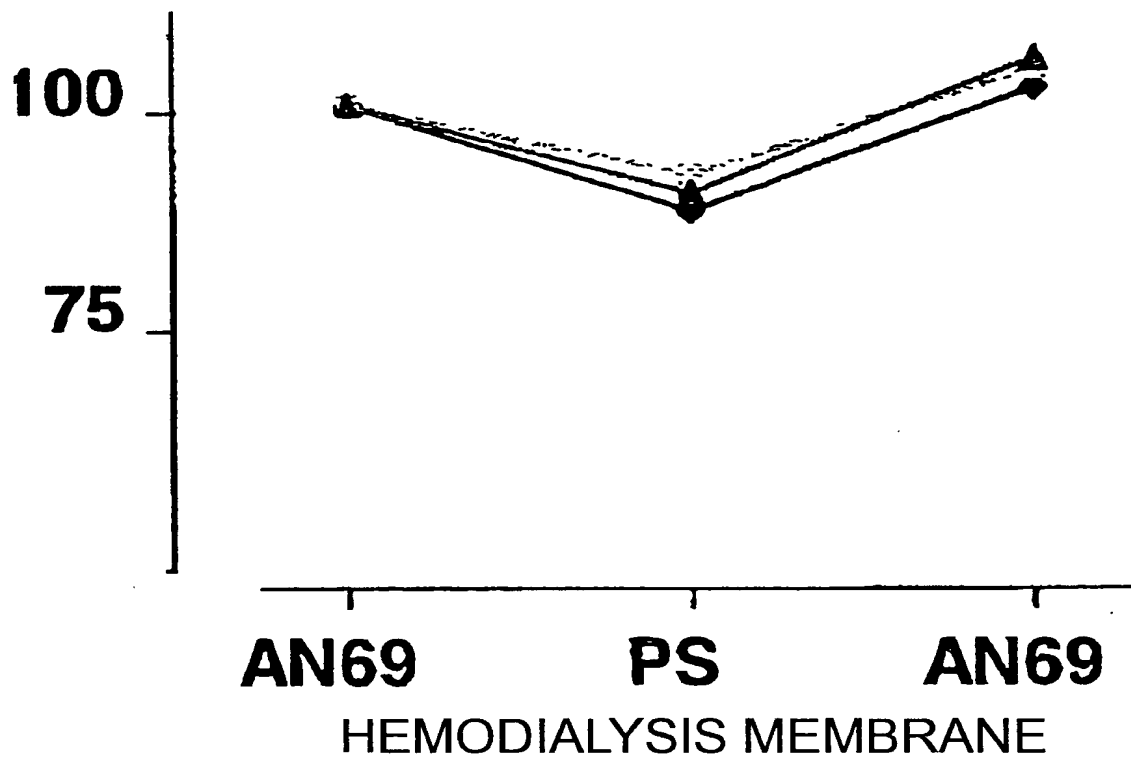
The suppression effect on the pentosidine level of blood  
35 plasma in dialysis patients by the incubation with beads on which a carbonyl compound-trapping agent had been immobilized is shown.



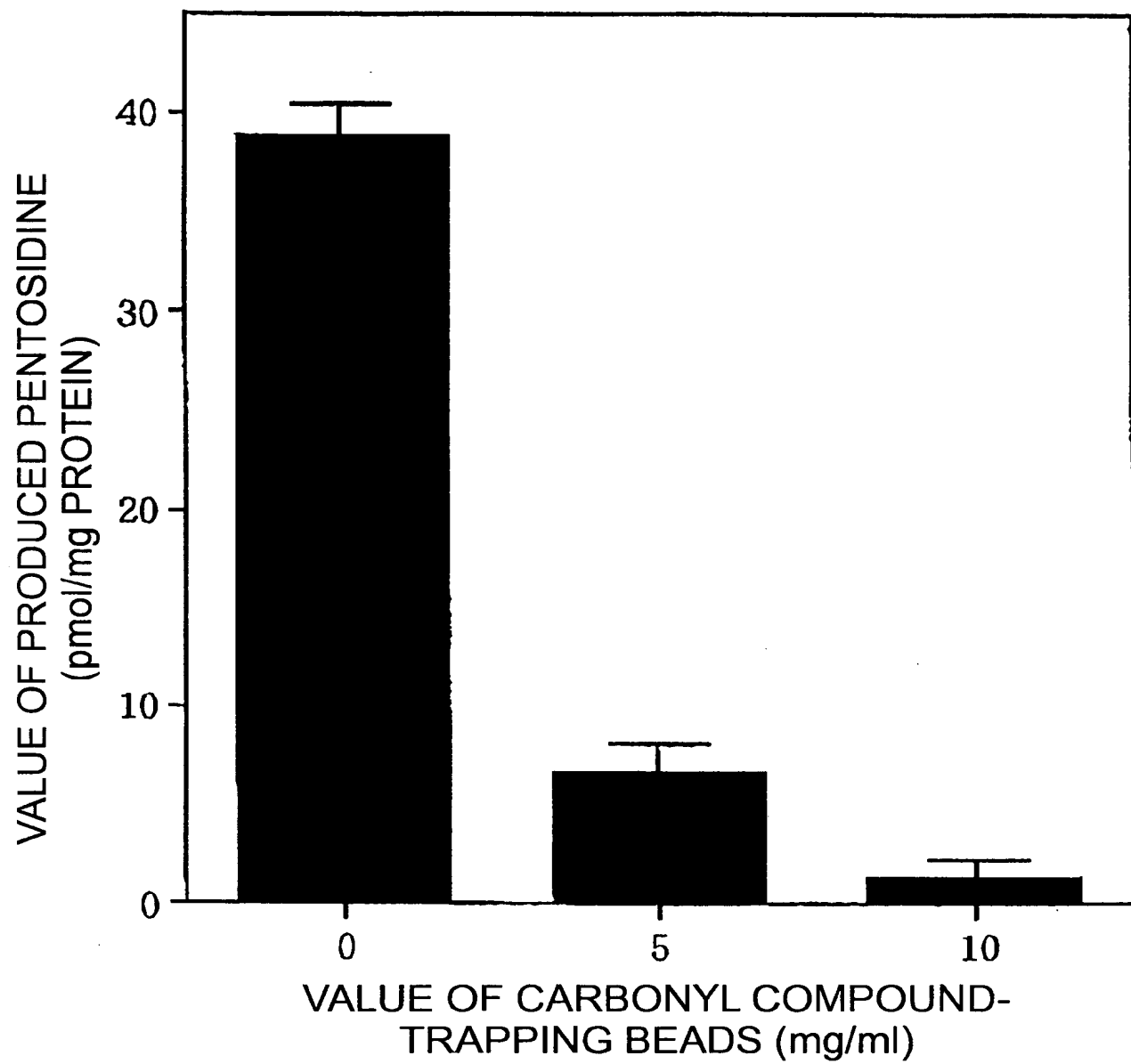
[Document Name] Drawings

[Fig. 1]

PROTEIN-BOUND PENTOSIDINE  
(% OF INITIAL VALUE)



[Fig. 2]



[Document Name] Abstract

[Abstract]

[Problems to Be Solved] An objective is to improve carbonyl stress state in hemodialysis patients.

5 [Means to Solve the Problems] A carrier to which a carbonyl compound-trapping agent is immobilized is contacted with a patient's blood. Thereby, carbonyl compounds are effectively removed from the patient's blood, and thus, damages from carbonyl compounds can be reduced.

10 [Selected Drawing] None